THE ENUMERATION OF BACTERIOPHAGES IN WATER AND SEWAGE

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INTRODUCTION

Since bacteriophages ('phages) are viruses, it has been suggested that certain of them may be useful as indicators of human enterovirus survival in water and sewage (2). Escherichia coli (E.coli) 'phages have been found to be present in water samples containing Coxsackie viruses (4).

A study by Kelly et al (3) compared the resistance of enteroviruses to that of 'phages, and found it to be similar.

The advantage of employing 'phage indicators, rather than the enteroviruses themselves, is that the former are relatively simple to isolate, culture and maintain, whereas study of the latter requires expensive and highly skilled techniques.

With a view to the possible use of 'phages for the monitoring of water and/or sewage for virus content, two methods for the enumeration of 'phages in water samples were investigated. These methods are particularly applicable to laboratory scale investigations into the efficiency of virus removal in certain water and sewage treatment procedures. One method (6) involves enumeration of 'phage by a Millipore filter (MF) technique, the other (5) by means of a most probable number (MPN) method. Both tests can be completed in 24 hours.

MATERIALS AND METHODS

BACTERIOPHAGES

The 'phages used were those of the strain B of E.coli, which was kindly supplied by the Department of Bacteriology, University of Toronto. The bacterial culture was maintained on nutrient agar slants at room temperature, transferred bi-weekly.

The 'phages were isolated from raw sewage according to the method given by Adams (1); they were not identified according to type, but purified preparations of each 'phage were obtained and stored at 4°C until required. The initial titrations of the 'phage preparations were made by the agar layer technique (1).

MEDIA

Difco Bacto nutrient agar, nutrient broth and m-Endo medium were used throughout, prepared as directed. The 'phage assay broth (PAB) consisted of: - Difco Bacto nutrient broth-8.0 gm., NaCl-5 gm. dissolved in 1 litre of distilled water. For 'phage assay agar (PAA) the PAB plus 15 gm. agar per litre was employed. The PAA and PAB were sterilized for 15 minutes at 15 psi; agar plates were dried in the incubator prior to use.

EXPERIMENTAL

a) MEMBRANE FILTER METHOD

The bacteriophage preparation was diluted in tenfold steps, in dilution water, and 1 ml. aliquots of these dilutions were added to 100 ml. amounts of distilled water, to yield approximately 10^4, 10^3, 10^2, 10 and 1 plaque forming unit (pfu) per 100 ml. sample.

The method (6) employed was as follows. To each sample was added about 10^9 cells of E.coli B, and the sample was stirred 5 minutes at room temperature at about 45 rpm. The sample was then filtered through a 0.45u MF; the filter was cultured on a pad soaked with m-Endo medium in a Petri dish at 35° overnight. The E.coli B culture was prepared by inoculating nutrient broth from an agar slant grown overnight and incubating, with shaking, for 5 hours at 34°C. The approximate yield of organisms at this point would be 10^8 per ml., and 6 to 10 ml. of such a culture would be added to each sample to be filtered.

After incubation, the plaques appearing in the confluent growth of E.coli B on each filter were counted, using a stereo-microscope. The counts were compared to the actual number of pfu's per 100 ml at each dilution. Preliminary experiments, as had the work of Loehr and Schwegler (6), showed that the MF titres were lower than those of the agar layer technique by a factor of approximately 20.

b) MOST PROBABLE NUMBER METHOD

The same bacteriophage samples as in a), containing 10^4, 10^3, 10^2, 10 and 1 pfu per 100 ml. were used. From each sample, the following tubes were set up according to Kott (4):-

5 x 10 ml. PAB double strength medium + 10 ml. sample

5 x 10 ml. PAB single strength medium + 1 ml. sample

5 x 10 ml. PAB single strength medium + 1 ml. 1/10 dilution of sample.

The tubes were incubated overnight at 35°C after the addition of 0.1 ml. of E.coli B suspension to each tube. The suspension was prepared by washing the overnight growth of E.coli B off a nutrient agar slant with about 7 ml. of nutrient broth.

A loopful of culture was then taken from each assay tube and placed on a PAA plate, freshly seeded with E.coli B. The appearance of a plaque on the plate, after about 6 hours' incubation at 35°C, indicated the presence of 'phage in the tube from which the loopful of culture was taken. The proportion of 'phage-positive tubes could thus be found and, using the standard coliform MPN table, the most probable number of 'phage particles in 100 ml. of sample was determined.

A number of lagoon, sewage and water samples were examined by both methods for the presence of E.coli B'phage. All such samples, with the exception of the water samples, were clarified by filtration through fibreglass. Sewage samples were normally tested undiluted and at a dilution of 1/10.

RESULTS

The 'phage preparation used in the initial tests had a titre of 9.0×10^9 pfu per ml. by the agar layer method. The results of three separate enumberations of this preparation by the MF and MPN methods are compared to the actual titre in Talbe 1. The results of the examination of lagoon and sewage samples are shown in Table 2.

CONCLUSIONS

As can be seen from the tables, the MPN method gives a titre which corresponds very closely to the actual titre; it is able to detect levels of 'phage of 9 pfu per 100 ml. and possibly less. It is also independent of the turbidity of the sample employed. The MF method however, unless the somewhat arbitrary factor of 20 is applied, gives a considerably lower titre and is able to detect 'phage only in the 1 pfu per ml. range. This is possibly due to loss of 'phage through the filter. The latter method also requires that a fairly good plaque-forming 'phage be present or the plaques formed on the filter are not sufficiently large to be dectectable; furthermore, it is not possible to filter sufficiently large samples of waters or sewage high in turbidity, before the filter becomes clogged.

Although only a few studies were carried out with field samples, the MPN method would appear to be the one of choice, although the plaques formed on the plates are sometimes obscured by an overgrowth of other organisms present in the sample. The presence of a plaque is determined by a halo effect around the dense growth, rather than by a complete clearing. This effect might possibly be overcome by the use of some type of selective medium, which would tend to decrease the growth of contaminants.

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TABLE 1

Comparison of the titres of E.coli B bacteriophage in Water Samples Obtained by the MPN and MF Techniques

Actual No. pfu per 100 ml*	pfu per 100 ml				
	MPN			MF	
		95% conf. limits	Average		Average
0	0 0 0	0 0 0	0	0 0 0	0
0.9	NT** 0 0	NT 0 0	0	NT 0 0	0
9.0	NT 5 2	NT <0.5-13 <0.5-7	3.5	NT 0 0	0
90.0	130 34 22	35-300 12-93 7-67	62	8 2 4	5
900.0	542 918 918	180-1400 300-3200 300-3200	793	98 29 37	55
9000.0	>5800 >5800 >5800	-	-	>600 235 290	-

* - based on the agar layer titre
** - not tested

TABLE 2

Enumeration of Bacteriophage in Field Samples by The MPN and MF Methods

Sample	pfu per 100 ml	
	MPN	MF
Sewage 1	>5800	76
Sewage 1 1/10	175	2
Sewage 2	221	294
Sewage 2 1/10	79	4
Lagoon effluent (Bradford)	172	28
Mixed liquor Dixie STP	278	1
Tap Water	0	0

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